TECHNICAL COMMENT

BIOPHYSICS

Comment on "Extreme electric fields power catalysis in the active site of ketosteroid isomerase"

Aditya Natarajan,[1] Filip Yabukarski,[1] Vandana Lamba,[1] Jason P. Schwans,[2] Fanny Sunden,[1] Daniel Herschlag[1]*

Fried *et al*. (Reports, 19 December 2014, p. 1510) demonstrated a strong correlation between reaction rate and the carbonyl stretching frequency of a product analog bound to ketosteroid isomerase oxyanion hole mutants and concluded that the active-site electric field provides 70% of catalysis. Alternative comparisons suggest a smaller contribution, relative to the corresponding solution reaction, and highlight the importance of atomic-level descriptions.

We were excited to see the data of Fried *et al.* (*1*) demonstrating a strong correlation between reaction rate and the stretching frequency of the carbonyl group of a product analog bound to a series of ketosteroid isomerase (KSI) oxyanion hole mutants. These data were interpreted in terms of a model, calibrated using vibrational data and molecular dynamics simulations in a series of solvents, which led to the conclusion that the active-site electric field generated by the oxyanion hole and surrounding groups accounts for 10^5-fold rate enhancement and 70% of the observed catalysis. Based on these findings, it was suggested that electrostatic forces are the dominant contributor to catalysis.

Below, we note that the conclusion of a dominant contribution to KSI catalysis relies on comparison to a hypothetical enzyme that provides zero electric field at the position of the carbonyl group and would not hold for a comparison to the corresponding reaction in aqueous solution. The accompanying analysis leading to an estimate of the rate advantage from positioning of KSI's general base is similarly affected. Finally, we note that electrostatic stabilization requires and is linked to positioning of the groups responsible for that stabilization.

Conservative mutations, such as the $Tyr^{16}Phe$ mutation in the construct employed by Fried *et al.*, often transmute a polar group to a nonpolar group and generate an apolar or hydrophobic environment that is less favorable toward charge accumulation than the polar environment in aqueous solution, and are inhibitory as a result (*2*). Thus, a "conservative" mutation of the dominant

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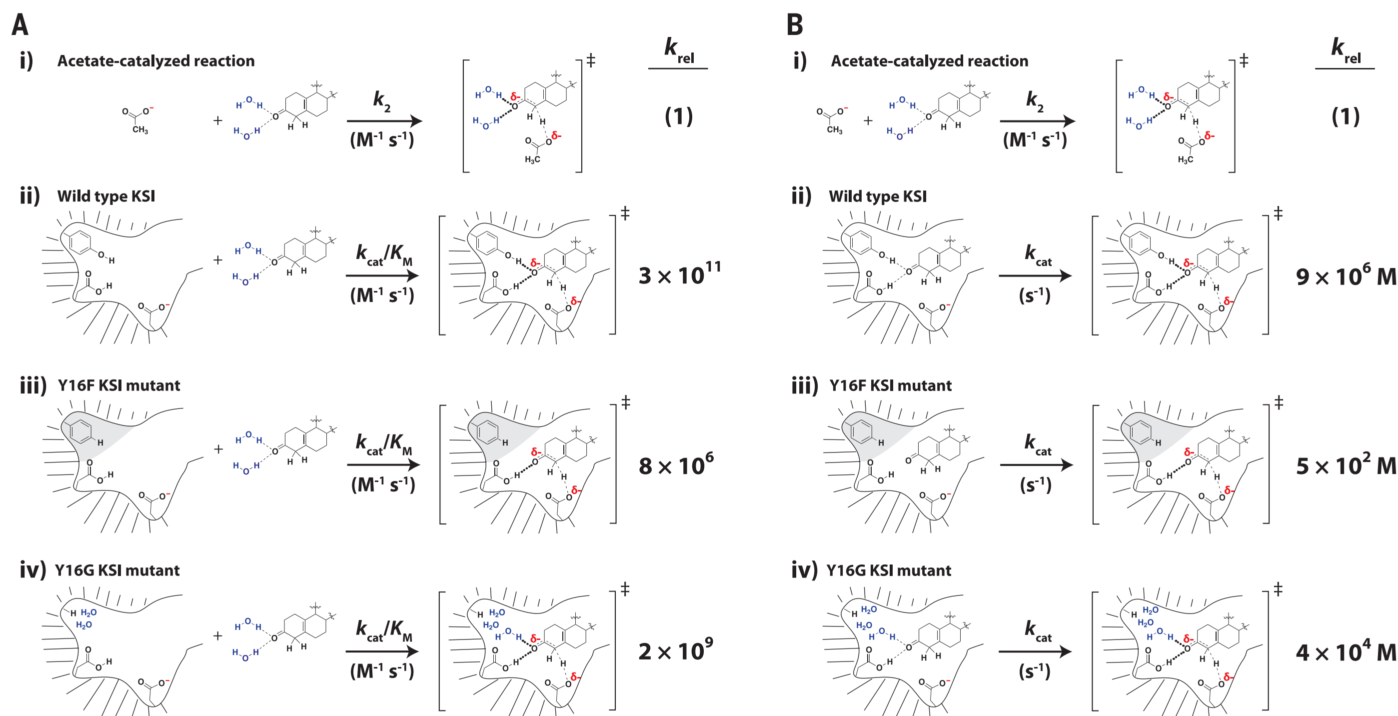

Fig. 1. Rate enhancement provided by different KSI variants relative to the rate of the acetate-catalyzed reaction. Rate enhancement provided by different KSI variants relative to the rate of the acetate-catalyzed reaction under (**A**) subsaturating and (**B**) saturating conditions. (i) Reaction between substrate and acetate in aqueous solution. (ii) Reaction between substrate and wild-type KSI. (iii) Reaction between substrate and KSI with a conservative $Tyr^{16}Phe$ mutation that replaces the oxyanion hole hydrogen-bond donor Tyr^{16} with a hydrophobic environment. (iv) Reaction between substrate and KSI with a $Tyr^{16}Gly$ mutation, more drastic than the $Tyr^{16}Phe$ mutation above. The units of each rate constant are shown in parentheses under the corresponding reaction arrow. Values were computed from rate constants for variants of KSI from *Pseudomonas putida* tabulated in table S2 in Kraut *et al*. (*2*) and correspond to the substrate 5(10)-estren-3,17-dione, because a chemical step is rate-limiting for reaction of this substrate (*9*). Similar rate enhancements are provided for reactions with the substrate 5-androstene-3,17-dione referred to by Fried *et al*., although a nonchemical step is partially rate limiting for this substrate (*9*, *10*).

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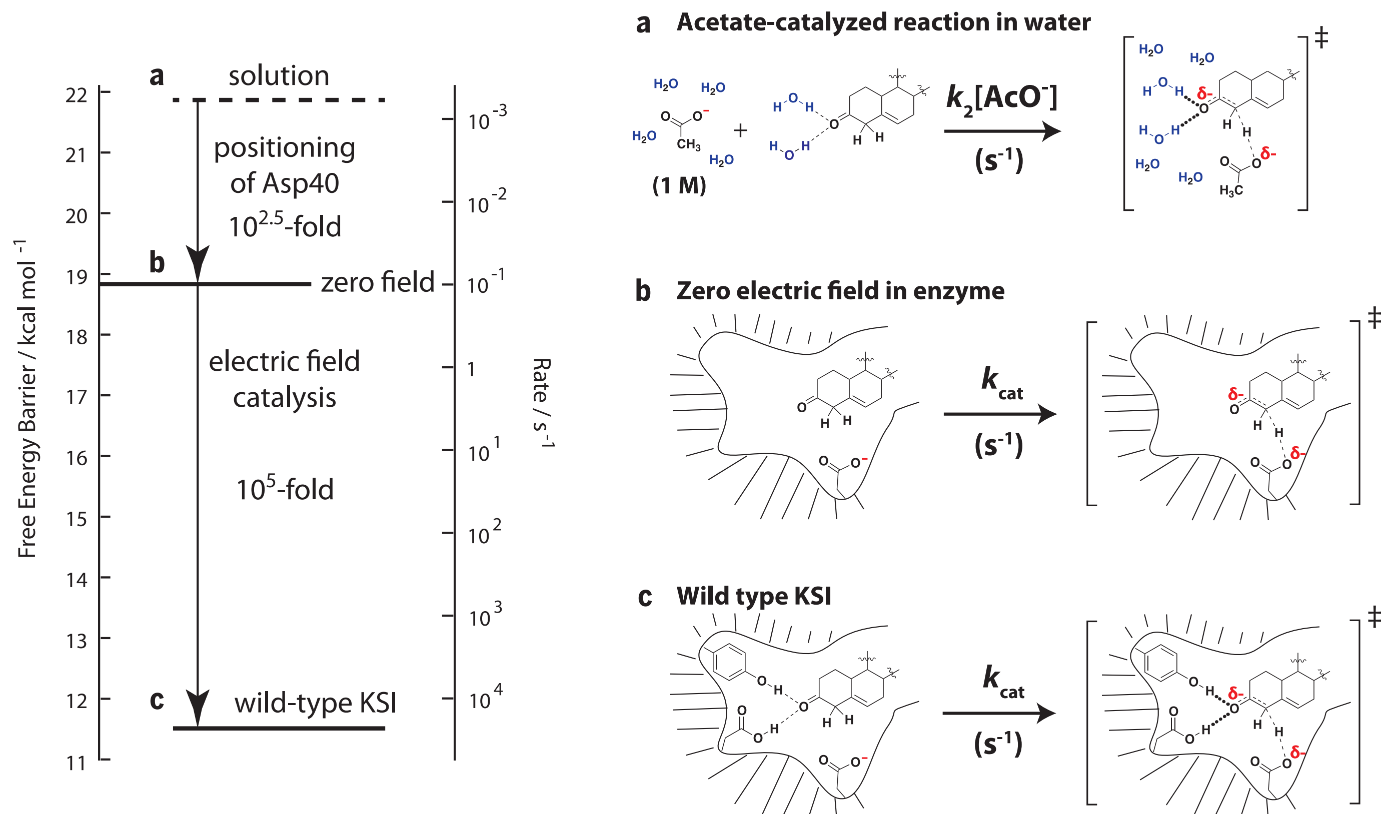

Fig. 2. Reactions used by Fried *et al*. to estimate the fraction of the overall catalytic power from the electric field on the carbonyl group. The left side of the figure is reproduced from Fried *et al*. with the reactions annotated by the lowercase letters and a schematic of each corresponding reaction shown on the right. The reactions are depicted schematically on the right and are as follows: (a) Nonenzymatic reaction between acetate and substrate in aqueous solution. Because this is a second-order process whose reaction rate depends on acetate concentration, Fried *et al*. used the observed rate constant at 1 M acetate (so that the calculated rate enhancement of $10^{2.5}$ is unitless but will vary with different concentrations of acetate used as the reference reaction). (b) Reaction with substrate bound to an enzyme that provides zero electric field at the carbonyl (and the same contribution from the general base as in wild-type KSI). (c) Reaction with substrate bound to wild-type KSI.

KSI oxyanion-stabilizing residue (*3*), such as $Tyr^{16}Phe$, can exaggerate the catalytic contribution of active-site hydrogen-bonding groups relative to aqueous solution (Fig. 1A, reaction iii). Indeed, KSI variants with more extreme ablation of Tyr16 (e.g., $Tyr^{16}Ser$, Ala, or Gly) are two orders of magnitude more active than the Tyr16Phe variant (Fig. 1A, reaction iv) (*2*), suggesting that replacing the hydrophobic Phe residue with what seems to be a relatively disordered water molecule is much less deleterious (*2*). In this mutant, only ~20% (log scale) of the catalytic power is lost and ~10^9-fold catalysis remains, suggesting that the oxyanion hole may not be the dominant contributor to KSI catalysis

Below, we lay out more explicitly the comparisons used by Fried *et al.* and demonstrate that there is likely more catalysis from the positioned general base than estimated by these authors, thereby helping to clarify the relative contributions of catalytic mechanisms and the underlying comparisons used to derive them.

Figure 3C from Fried *et al.* proposes a full accounting of catalysis, assigning a modest catalytic contribution from general base positioning and a dominant contribution from the enzyme's electric field. Figure 2 reproduces figure 3C in Fried *et al.*, annotated with the corresponding reactions that were used by these authors to estimate the catalytic contributions. Three reactions were considered:

a. Nonenzymatic reaction in aqueous solution between 1 M acetate and substrate.

b. Substrate bound to a hypothetical enzyme that provides zero electric field at the position of the carbonyl group.

c. Substrate bound to wild-type enzyme.

Fried *et al.* estimated the contribution from the positioned general base from comparison of reactions **a** and **b**, but these reactions have two differences, and thus, the comparison does not isolate the contribution from the positioned general base. Specifically, reaction **a** takes place with free general base (acetate ion) in aqueous solution, whereas reaction **b** takes place with positioned general base in an enzyme with a zero electric field at the oxyanion hole (Fig. 2). As noted above, a hydrophobic or zero-field oxyanion hole environment would be inhibitory relative to an aqueous environment, as also implied by the comparisons of figure 3, A and B, of Fried *et al.* Stated another way, charge localization is more difficult upon removal of the aqueous surroundings and placement in a zero field or hydrophobic environment. Thus, the effect from the positioned general base appears to be underestimated by the comparison of reactions **a** and **b**. Correspondingly, this result implies an overestimation of the catalytic effect from the oxyanion hole.

In addition, Fried *et al.* use the assumption that the oxyanion hole and general base contributions are independent to parse the catalytic contributions, although, as they note, there is no experimental evidence to support this assumption. The additivity of the catalytic features remains to be tested.

Fried *et al.* consider effects of positioning and electrostatics and state that "contrary to earlier views [(*4*, *5*)], electrostatic stabilization can be the more important of the two (Fig. 3C)." We emphasize that positioning is also important for electrostatic catalysis. As Fried *et al.* note elsewhere, "the active site achieves this large field by restricting H-bond conformations to those that are associated with the largest electric fields." If there were no positioning of the active site groups giving rise to the electric field, the fields would be lower, and if positioning of the oxyanion hole and surrounding residues were incorrect (e.g., backward with respect to substrate), the fields could even be inhibitory. The field is a consequence of

both the functional groups that are present and their positioning. This positioning arises from the folding of the protein, using favorable folding energy to orient and restrict the conformational mobility of these groups, and from binding of the substrate, in a pocket also formed due to folding of the protein (*6*–*8*). Turning to the substrate, if there were no pocket or if the substrate were sterically restricted from approaching the oxyanion hole, then there would be less or no catalysis; if the substrate were bound but positioned such that its carbonyl group faced away from the oxyanion hole, then the oxyanion hole and its surroundings would not contribute to catalysis. In summary, electrostatic catalysis, to be effective, requires positioning—proper positioning of the substrate via binding interactions into a pocket that is created via protein folding as well as proper positioning of enzymatic groups, again via protein folding and substrate binding, to make favorable electrostatic interactions in the reaction's transition state. Thus, catalytic contributions from electrostatics and positioning appear to be inextricably linked. Understanding this linkage, and catalysis, will likely require descriptions that extend beyond measures of apparent electric fields to atomic-level descriptions and models, including the multiple states present in the ensemble of an enzyme-substrate complex and the reaction probability from each state.

Another future challenge will be to understand the extent to which functional groups near and far from the active site contribute to the electric field perceived at the substrate carbonyl and how much they contribute to catalysis. Fried *et al.* (*1*) cite the observation of a nonzero field in the Tyr^{16}Phe mutant to conclude that "a substantial electrostatic field contribution also arises from the environment fashioned by the enzyme scaffold." An alternative model that remains to be tested is that the nonzero field in this mutant arises largely from Asp^{103}, the other oxyanion hole hydrogen-bond donor. The observation of a negligible electrostatic field effect from mutation of the Asp^{40} general base to Asn suggests a limited propagation distance (*1*).

Vibrational measurements, like those elegantly presented by Fried *et al.*, provide a powerful means to compare enzyme variants, and the observed vibrational properties can be compared to predictions from computation to assess those models. For example, Fried *et al.* note that molecular dynamics simulations with KSI did not reproduce the electric fields calculated from the experimentally observed vibrational frequencies [supplementary text 4 of (*1*)], providing a strong indication of features or factors lacking in the computation model, in its implementation, or in its underlying physical forces. Analogously, whether hydrogen-bond interactions can be quantitatively and accurately modeled as field effects or correlate with field effects and require more sophisticated atomic-level models remains to be determined. Regardless, vibrational data, as obtained by Fried *et al.* and others, provide an incisive window into the active site that will help test, develop, and refine increasingly accurate and predictive models for enzymatic catalysis.

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TECHNICAL COMMENT

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Comment on "Extreme electric fields power catalysis in the active site of ketosteroid isomerase"

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Fried *et al*. (Reports, 19 December 2014, p. 1510) demonstrate electric field–dependent acceleration of biological catalysis using ketosteroid isomerase as a prototypic example. These findings were not extended to aqueous solution because water by itself has field fluctuations that are too large and fast to provide a catalytic effect. Given physiological context, when water electrostatic interactions are considered, electric fields play a less important role in the catalysis.

Fried *et al*. (*1*) report that the isomerization of 5-androstene-3,17-dione by ketosteroid isomerase (KSI) has a markedly higher (4×10^7 M) rate constant (k_{cat}) than in aqueous solution (k^{AcO}). The free-energy barrier for the reaction catalyzed by KSI is much lower because (i) the difference in effective acetate (COO^-) concentration (ΔG_S) is higher for KSI than for k^{AcO}; (ii) different electric-field contributions on the C=O bond ($\Delta G_{C=O}$); and (iii) hydrogen abstraction by the carboxyl group (ΔG_H), as shown in Fig. 1. Thus,

$$\Delta G_S + \Delta G_{C=O} + \Delta G_H = -RT\ln(4 \times 10^7) = -10.5 \text{ kcal mol}^{-1} \quad (1)$$

Fried *et al*. also report that the free-energy barrier for the rate-limiting enolate transition catalyzed by KSI is 11.5 kcal mol^{-1}, compared with 18.8 kcal mol^{-1} in a nonpolar environment where the electric field is 0. On this premise, KSI contributes an electric field of 7.3 kcal mol^{-1} to its free-energy-barrier reduction when compared with the nonpolar environment. This model assumes that bulk water confers no electric power toward catalysis because it has field fluctuations that are too wide and fast compared with the narrow infrared shifts that are evident in KSI and its active-site mutants. Thus, $\Delta G_{C=O}$ is −7.3 kcal mol^{-1}, ΔG_S is approximately −3.2 kcal mol^{-1}, and ΔG_H is 0, because the model does not take into account the effect of hydrogen abstraction on the free-energy barrier.

Life on earth depends on water and the hydrogen bonds that it forms in biological systems. Water can accelerate reactions by more than 10^{10}-fold (>13.8 kcal mol^{-1} of free-energy-barrier reduction) when atoms in transition states become more charged than in ground states (Fig. 1). Such hydrogen bonds potentiate catalysis in a number of ways that are also directly relevant to the isomerization of 5-androstene-3,17-dione by KSI mutants that contain active-site cavities sufficiently large for water to interact with C=O, as suggested by Kraut *et al*. (*2*). For example, because water forms electrostatic interactions with the negatively charged oxygen atom of the C=O group in 5-androstene-3,17-dione, these forces stabilize transition states as the oxygen atom is more negatively charged and reduce the free-energy barrier of the isomerization reaction by ~4.0 kcal mol^{-1}.

The broad line width of C=O in 19-nortestosterone measured in aqueous solution indicates that electrostatic interactions between water and C=O adopt diverse conformations. A thermodynamic cycle shows to what extent broad electric fields of water can reduce the free-energy barrier for the isomerization reaction of 5-androstene-3,17-dione (Fig. 2). ΔG_{rig} represents the free-energy-barrier reduction by well-oriented active-site–associated water, which is expected to contribute a larger C=O spectral shift than in aqueous solution. Thus, ΔG_{rig} is less than −4 kcal mol^{-1}, based on the infrared spectra for C=O in nonpolar solvent, water, and KSI. A more accurate calculation of ΔG_{rig} (−4.8 kcal mol^{-1}) is derived from the analysis of free-energy barriers during the isomerization of 5-androstene-3,17-dione by the KSI $Tyr^{16}Ser$ mutant, where water can interact with C=O (13.6 kcal mol^{-1}), versus $Tyr^{16}Phe$ (16.0 kcal mol^{-1}), where water interactions are absent (*1*, *2*). This is supported by the observation that the infrared spectral shift of 19-nortestosterone bound to the $Tyr^{16}Ser$ mutation is narrow (*1*). From this, ΔG_{sol} is the free-energy-barrier reduction by water C=0 interactions and can be obtained by

$$\Delta G_{sol} = -4.8 + \Delta G_1 - \Delta G_2 \quad (2)$$

Both ΔG_1 and ΔG_2 are greater than 0 at room temperature, just as ΔG for the transition from water to ice is greater than 0. Because exact values for ΔG_1 and ΔG_2 are not available, we estimated ΔG_1 by assuming that water-C=O and water-water interactions are comparable. ΔG_1 is the maximum free energy of reorganization for the reference reaction and is close to the free-energy change of the process in which one hydrogen bond of a water molecule is fixed and the water molecule can still rotate freely around the hydrogen bond. At 273.2 K, ΔG, ΔH, and ΔS from water to ice are 0, −1.44 kcal mol^{-1}, and −5.26 cal mol^{-1} K^{-1}, respectively. As one mole of water molecules in ice contains two moles of hydrogen bonds, and constrained water in the cycle can rotate in one direction, ΔH_1 and ΔS_1 are −0.72 kcal mol^{-1} and −4.38 cal mol K^{-1}. $\Delta G_1 = 2 \times [-0.72 - 298 \times (-4.38)/1000] = 1.17$ kcal mol^{-1}. Thus, the free-energy-barrier reduction by water is $\Delta G_2 + 3.63$ kcal mol^{-1}. Because ΔG_2 is similar to ΔG_1, ΔG_{sol} is closer to −4.0 kcal mol^{-1}, which is estimated based on the C=O spectral shift in aqueous solution. Electrostatic interactions between the C=O group and water therefore contribute ~4.0 kcal mol^{-1} to the free-energy-barrier reduction for the reference reaction in water. Thus, the electrostatic contribution of the C=O group to free-energy-barrier reduction for the reaction catalyzed by KSI versus the reaction in water is 3.3 kcal mol^{-1} (Fig. 2).

The origin of ΔG_H is desolvation of active-site Asp^{40}. In aqueous solution, the COO^- group is completely solvated and at least one water molecule is removed for COO^- to abstract the α-hydrogen atom, whereas in KSI, no water molecules are removed for Asp^{40} to abstract the hydrogen atom (Fig. 1). The crystal structure of *Pseudomonas putida* KSI mutant $Asp^{40}Asn$ complexed with androsten-3-betal-ol-17-one (*3*) shows that the oxygen atom involved in abstracting the α-hydrogen is desolvated and surrounded by hydrophobic groups (Fig. 2), indicating that the desolvation process does not occur from the ground to transition state catalyzed by KSI. Rather, desolvation of Asp^{40} occurs during substrate binding to KSI, supported by the observation that analog binding affinity increases by ~2 orders of magnitude for the $Asp^{40}Ala$ mutation compared with wild-type (*4*). Moreover, electrostatic interactions with the COO^- group decrease as the reaction proceeds because the oxygen atoms of COO^- become less negatively charged, thereby increasing the free-energy barrier. This free-energy-barrier increment for the reference reaction—in which the COO^- group interacts with three water molecules (Fig. 1)—is larger than that for the reaction in KSI, in which the COO^- group interacts with Trp^{120} (Fig. 2). Thus, desolvation of Asp^{40} can reduce the free-energy barrier to a meaningful extent. Similar cases exist in organic reactions in which the desolvation of anions can accelerate reactions dramatically (Fig. 1).

In summary, the relative free-energy contribution of KSI's catalytic power includes electric-field and desolvation effects, as well as general base positioning. The contribution of the electric-field effect compared to that in water is 3.3 kcal mol^{-1} and accounts for ~31.4% of KSI's catalytic speed-up relative to the uncatalyzed reference reaction

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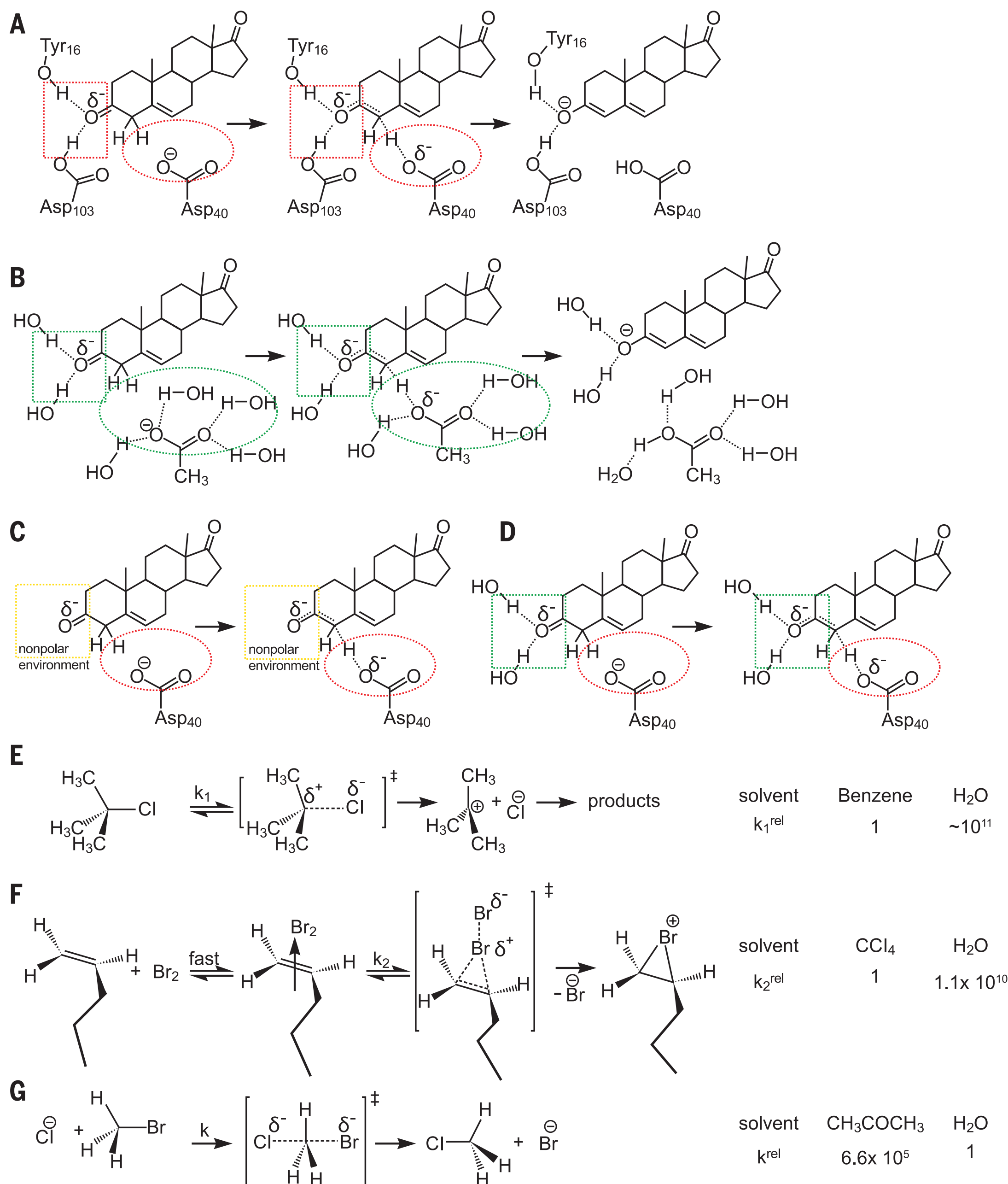

Fig. 1. Isomerization of 5-androstene-3,17-dione and the effects of water on free-energy barriers. The chemical mechanism for the first step of the isomerization reaction catalyzed by KSI (**A**) and the uncatalyzed reaction in aqueous solution (**B**). The two reactions differ in (i) electrostatic interactions of the C=O group (squares) and (ii) the carboxyl group abstracting the α-hydrogen (circles). (**C**) Ground and transition states for the isomerization reaction in which the electric field exerted on the C=O bond is zero (nonpolar environment), whereas the carboxyl group abstracting the α-hydrogen is similar to (A). (**D**) Ground and transition states for the isomerization reaction in which the electric field exerted on the C=O bond is the similar to that of aqueous solution in (B), whereas the carboxyl group abstracting the α-hydrogen is similar to (A). (**E** and **F**) Water accelerates reactions in which the atoms are more charged in transition than ground states (*5*). (**G**) Desolvation accelerates reactions in which the atoms are less charged in transition than ground states (*5*, *6*).

in aqueous solution. However, it is worthwhile emphasizing that the overall energy gain of the reaction is distinct from that contributed by electrostatic interactions of C=O. Regardless of the overall gain for the reaction in aqueous versus hydrophobic environments, electrostatic interaction of C=O with water for the reference reaction contributes toward the free-energy-barrier reduction. Exact contributions of desolvation (ΔG_H) and general base positioning (ΔG_S) to free-energy-barrier reduction cannot be calculated based on the information provided in this paper.

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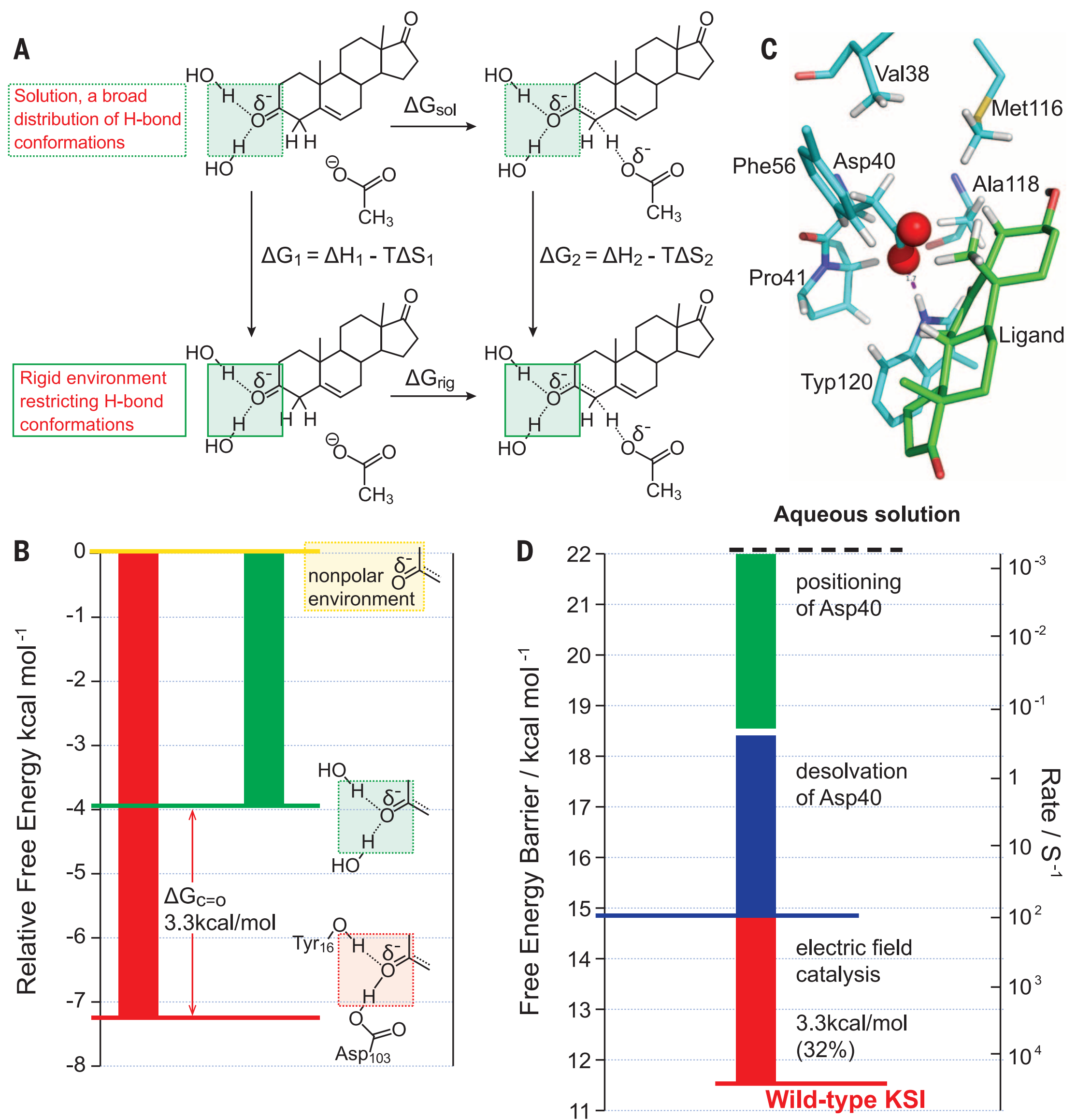

Fig. 2. Contribution of active-site electric fields and desolvation of Asp[40] to KSI's catalytic power. (**A**) Thermodynamic cycle showing the electrostatic contributions of the C=O group to the free-energy-barrier reduction (ΔG_{sol}) for the isomerization of 5-androstene-3,17-dione in water solution. (**B**) Relative electrostatic contributions of the C=O group to free-energy-barrier reduction in a nonpolar environment, in water and KSI. The free-energy-barrier reduction in KSI is 3.3 kcal mol^{-1} more than that in aqueous solution. This represents the contribution of electric fields to the catalytic power of KSI because the catalytic power of KSI is estimated based on the uncatalyzed reaction in aqueous solution. (**C**) Crystal structure of KSI mutant Asp[40]Asn complexed with androsten-3-betal-ol-17-one (Protein Data Bank, 1E3R) (*3*). The nitrogen atom of Asn[40] is changed to an oxygen atom. Red spheres represent oxygen atoms of the carboxyl group. Hydrogen atoms that affect solvent-accessible areas of the oxygen atoms are shown. The oxygen atom abstracting α-hydrogen is surrounded by hydrophobic groups. The other oxygen atom forms a hydrogen bond with Trp[120]. (**D**) The relative contribution of KSI's catalytic power. The electric field contributes ~32%, which is markedly less than the combined base positioning and desolvation of Asp[40], which contribute approximately 68%.

TECHNICAL RESPONSE

BIOPHYSICS

Response to Comments on "Extreme electric fields power catalysis in the active site of ketosteroid isomerase"

Stephen D. Fried* and Steven G. Boxer†

Natarajan *et al*. and Chen and Savidge comment that comparing the electric field in ketosteroid isomerase's (KSI's) active site to zero overestimates the catalytic effect of KSI's electric field because the reference reaction occurs in water, which itself exerts a sizable electrostatic field. To compensate, Natarajan *et al*. argue that additional catalytic weight arises from positioning of the general base, whereas Chen and Savidge propose a separate contribution from desolvation of the general base. We note that the former claim is not well supported by published results, and the latter claim is intriguing but lacks experimental basis. We also take the opportunity to clarify some of the more conceptually subtle aspects of electrostatic catalysis.

Although the active site of ketosteroid isomerase (KSI) exerts a very large electric field (magnitude close to 150 MV/cm) onto its bound substrate, the substrate experiences a rather large electric field (magnitude 80 MV/cm, on average) when interacting with water in aqueous solution. A key point raised by Natarajan *et al.* (*1*) and Chen and Savidge (*2*) in their Comments is that bulk water would exert a catalytic effect on KSI's reaction proportional to its electric field. Chen and Savidge further imply that polar reactions are always accelerated by a polar medium. These suggestions reflect fundamental misunderstandings. Marcus theory teaches us that in reactions (such as KSI's) during which dipoles reorient and charges move, a polar solvent can actually have an inhibitory effect because there is an energetic cost (the reorganization energy) imposed by the requirement for the solvent sphere to forfeit the conformation that stabilizes the reactant's charge configuration to adopt a conformation that stabilizes the transition state's (TS's) charge configuration (*3*). An electric field (regardless of magnitude) can only have a catalytic effect by the model in figure 1B of our paper [Fried *et al.* (*4*)], if it adopts an orientation that specifically stabilizes the TS—that is, it is preorganized (*5*). The observation of narrow C=O bands in all the KSI active sites studied suggests that KSI's active-site electric field is fixed and preorganized [see also (*6*)] and therefore capable of producing a catalytic effect proportional to field magnitude. Solvent reaction fields (such as in bulk water) are not preorganized because they stabilize the reactant's (and not the TS's) charge configuration and have large fluctuations. The importance of this distinction is evidenced by the fact that several fundamental polar reactions are faster in the gas phase than in aqueous solution (*7*). Therefore, we do not think a priori that water itself will provide a catalytic effect due to its electric field relative to the gas phase, and for this reason, we counted the electric field of the KSI active site in full to estimate its contribution to catalysis.

Natarajan *et al.* are correct to emphasize that the electric field C=O experiences is the result of both the residues that create the field and those that position the steroid ligand within it; indeed, the "electric field picture" (*8*) illuminates why key hydrogen-bond–donating residues cooperate with positioning residues to confer maximal catalytic effect. To be clear, we reserve the term "chemical positioning" to refer to the positions of components that participate in chemistry (i.e., breaking and forming of bonds); this should be largely separable from electrostatic catalysis, which depends on the positions of atoms that define the environment in which the reaction occurs but do not necessarily participate in the reaction. Natarajan *et al.* suggest that chemical positioning provides the majority of KSI's catalytic effect. This suggestion seems unlikely to us, since several experiments conducted by Herschlag and colleagues that directly examined the effect of positioning Asp^{40} are consistent with our $10^{2.5}$-fold assignment: The introduction of mutations that misposition Asp^{40} (*9*, *10*) reduces activity by a factor of $10^{1.5}$ to 10^{3}. It would be very interesting to conduct "chemical rescue" studies (*11*) in which activity is restored to an Asp^{40}Gly mutant of KSI with exogenous acetate. Our assignment for the contribution of chemical positioning [figure 3C in (*4*)] could be tested by measuring the effective acetate concentration that provides an equivalent rate as wild-type KSI.

Natarajan *et al.* emphasize experiments on a collection of mutants of KSI in which the oxyanion hole and its environs are removed and partially replaced with small residues, leaving a water-filled cavity in its wake (part of which gets displaced by the substrate). These mutants reduce KSI's catalytic effect by 10^{3}-fold, and the authors take this to imply that electrostatic stabilization of C=O by KSI's active site provides a 10^{3}-fold effect relative to water. We disagree with this claim because, first, the active-site electric field arises not only from the oxyanion hole but also from the enzyme scaffold as a whole (so removing the oxyanion hole side chains would not remove all contributions to the active-site electric field). Second, it is unlikely that the water molecules trapped in the active site are analogous to bulk water, because they will be (partially) preorganized by the same enzyme scaffold that would otherwise organize Tyr^{16} and Asp^{103}. Enzyme design efforts have demonstrated that active-site waters (much like amino acid residues) can assist or impede catalysis depending on their positions, orientations, and dynamics (*12*, *13*). Specifically in the case of KSI, water dynamics in the active-site cleft near a substrate analog were found to be substantially different from those of bulk water, even for a rather exposed region of the cleft (*14*).

Chen and Savidge suggest the existence of an important third catalytic contribution from the placement of Asp^{40} in a nonpolar environment. Rephrasing their idea as we understand it, the concept is that just as C=O becomes more polar in the TS, and so would be stabilized by an active-site environment that exerts larger electric fields than water, the carboxyl group of Asp^{40} becomes less polar in the TS, and so would be less destabilized by an environment that exerts smaller electric fields than water. Such proposals would have to be tested by measuring the electric field on the carboxyl group across several mutants and seeing if it bears any relationship to rate. We would hesitate to assign this hypothetical effect a specific catalytic weight in the absence of experimental evidence, although in any event, it should still qualify under the heading of electrostatic catalysis.

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